Sequence requirements for proinsulin processing at the B-chain/C-peptide junction

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Proinsulin is converted into insulin by the action of two endoproteases. Type I (PC1/PC3) is thought to cleave between the B-chain and the connecting peptide (C-peptide) and type II (PC2) between the C-peptide and the A-chain. An acidic region immediately C-terminal to the point of cleavage at the B-chain/C-peptide junction is well conserved throughout evolution and has been suggested to be important for proinsulin conversion [Gross, Villa-Komaroff, Kahn, Weir and Halban (1989) J. Biol. Chem. **264**, 21486–21490]. We have here compared the precise role of this region as a whole and just the first acidic residue C-terminal to the point of cleavage in processing of proinsulin by PC3. To this end, several mutations were introduced in this region of human proinsulin (native sequence, B-chain RREAEDL C-peptide): RRPAEDL (C1Pro mutant); RRLAEDL (C1Leu mutant); RRL (C1–C4del mutant); RRE (del-C1Glu mutant). Mutant and native cDNAs were stably transfected into AtT20 (pituitary corticotroph) cells, in which PC3 is known to be the major conversion endoprotease, and kinetics of proinsulin conversion were studied (pulse–chase/HPLC analysis of proinsulin-related peptides). The results show that the acidic region following the B-chain/C-peptide junction is indeed important for PC3 cleavage at this site, and that the reduced cleavage observed for the C1–C4del mutant proinsulin can be partially overcome by replacing the acidic region with a single acidic residue (del-C1Glu mutant). Replacing only the first residue of the acidic region with leucine (C1Leu mutant) has no impact on conversion, whereas its replacement with proline (C1Pro mutant) almost completely abolishes cleavage at the B-chain/C-peptide junction without affecting that at the C-peptide/A-chain junction.

INTRODUCTION

Insulin, in common with many other hormones and proteins, is produced by post-translational endoproteolysis of a biosynthetic precursor, proinsulin [1–3]. Proinsulin is converted into insulin in the secretory granules of pancreatic β-cells [4–7]. Two endoproteolytic activities have been suggested to be responsible for this conversion [8]. Recently these activities have been shown to correspond to the endoproteases PC2 and PC3 (also called PC1) [9–11], two members of the mammalian family of subtilisin-like proteases related to the yeast Kex2 gene product [3,12–14]. Type-I endoprotease (PC3) cleaves on the C-terminal side of the pair of basic amino acids Arg^{31}-Arg^{32} linking the B-chain and connecting peptide (C-peptide) and type II (PC2) on the C-terminal side of Lys^{64}-Arg^{65} linking the C-peptide and the A-chain [8]. The C-terminal basic residues generated by such cleavages are then trimmed by carboxypeptidase E/H [15].

The presence of a pair of basic residues is a minimum requirement for conversion [16–18], but other structural domains are believed to participate in the presentation of such basic residues to the endoproteases [3,19,20]. The secondary structure around the cleavage site of many proproteins seems to be important in this context given that pairs of basic residues known to reside at sites of endoproteolytic cleavage are often in regions forming β-turns or immediately adjacent to such regions [21–23]. A stable local structure called the 'C/A knuckle' has been identified at the proinsulin C-peptide/A-chain junction by NMR studies and could be a recognition site for the endoprotease PC2 [24]. No analogous structure has been identified at the B-chain/C-peptide junction. The central region of C-peptide itself does not seem to be important for proinsulin processing as deletion of a large portion of this region (residues 38–62) still allows proinsulin to be quite efficiently processed when transfected in AtT20 cells [17].

The first four residues of the C-peptide constitute a highly acidic region which has been remarkably conserved throughout evolution [25]. Deletion of these four amino acids in rat proinsulin II was shown to prevent conversion of the mutant proinsulin, suggesting its involvement in this process [25]. However, in this particular proinsulin molecule, this deletion brings a proline immediately C-terminal to the cleavage site between the B-chain and C-peptide. Consequently the inhibition of the conversion could have been due to the presence of proline, a cyclic amino acid that could induce a conformational change at the cleavage site, rather than deletion of the acidic region *per se*. It was furthermore not possible in this previous study to distinguish between the two conversion intermediates, des-31.32- and des-64.65-split-proinsulin and intact proinsulin itself. The impact of the deletion on cleavage at the B-chain/C-peptide as opposed to the C-peptide/A-chain junction could thus not be addressed. Given the suggestion that there is a close interaction between these two junctions [17,18,26], this is of more than academic interest.

In the present study, the role of the C-peptide acidic region in proinsulin conversion has been re-evaluated. To this end, native and mutant human proinsulin cDNAs were stably transfected into AtT20 cells, a pituitary corticotroph cell line that has previously been shown to process exogenous prohormones, including proinsulin [27,28], in addition to the major endogenous product pro-opiomelanocortin. These cells have been shown to express PC3 at very high levels, but to express only very small amounts of PC2 [29–34]. This makes them suitable for the

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium containing 450 mg/dl glucose; IBMX, isobutylmethylxanthine; KRB, Krebs/Ringer/bicarbonate buffer.

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study of substrate specificity in the conversion of proinsulin by PC3.

MATERIALS AND METHODS

Materials

The expression vector pRSVhIns was generously given by Dr. H.-P. Moore, University of California, Berkeley, CA, U.S.A. T4 DNA ligase and restriction endonucleases were purchased from New England Biolabs (Beverly, MA, U.S.A.). [^{3}H]Leucine for labelling cells was from ARC (St. Louis, MO, U.S.A.), [α-[^{35}S]thio]ATP for sequencing from Amersham International (Amersham, Bucks., U.K.) and ^{125}I-insulin from Sodiag (Losone, Switzerland). G418 (Geneticin) and pig insulin antiserum for immunoprecipitation were purchased from Sigma. Modified T7 polymerase and sequencing reagents were from United States Biochemical (Cleveland, OH, U.S.A.). Lumaflow II scintillating cocktail was obtained from Lumac (Olen, Belgium). Both the antiserum and the human insulin standard for radioimmunoassay were from Novo-Nordisk (Bagsvaerd, Denmark). Culture medium came from Gibco Life Technologies (Paisley, Scotland, U.K.) and fetal calf serum from Inotech (Dottikon, Switzerland).

Mutagenesis

Mutations were introduced into proinsulin by uracil-DNA-mediated site-directed mutagenesis (kit from Boehringer-Mannheim, Rotkreuz, Switzerland). Proinsulin cDNA (from plasmid pRSVhIns) was cloned into the *Hin*dIII–*Bam*HI site of M13mp18 RF DNA in order to perform the mutagenesis. Mutated cDNAs were then subcloned into the *Hin*dIII–*Bam*HI site of the pRSV plasmid, transformed into competent bacteria DH5 and sequenced.

Cell culture

AtT20 (pituitary corticotroph) cells were grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air, in Dulbecco's modified Eagle's medium with 450 mg/dl glucose (DMEM), supplemented with 110 units/ml penicillin, 110 μg/ml streptomycin and 10% fetal calf serum.

Transfection and selection of stably transfected clones

Mutant or native pRSVhIns (140 μg) was co-transfected with 24 μg of pRSVNeo into AtT20 cells at low cell density by the calcium phosphate precipitation method [35]. Cells were incubated with the DNA for 5–6 h and then shocked with glycerol for 1 min. Stable transfectants were selected by culture in medium containing 0.5 mg/ml (active concentration) G418. Insulin-producing clones were screened by radioimmunoassay [36] for (pro)insulin-like immunoreactivity in media taken after a 1 h incubation of the cells under basal conditions in DMEM containing 10 mM Hepes and 0.5% BSA and after a second 1 h incubation under stimulatory conditions using the same medium supplemented with 1 mM isobutylmethylxanthine (IBMX) and 10 μM forskolin to raise intracellular cyclic AMP. Two clones of each transfection (except for the del-C1Glu mutation for which only one clone expressing appropriate levels of immunoreactive insulin could be studied) were selected for further study.

Analysis of steady-state content of (pro)insulin-like material

Cells were grown to near-confluence in 10 cm-diameter Petri dishes, washed once with PBS, extracted into 5 ml of 1 M acetic acid/0.1% BSA, sonicated and centrifuged at 11000 *g* at 4 °C for 15 min. The supernatants were prepurified on C_{18} Sep-Pak cartridges (Waters Millipore, Bedford, MA, U.S.A.) [37] and analysed by reverse-phase HPLC by established procedures [28,38]. The fractions (1 ml) were collected in borosilicate tubes containing 100 μl of 0.5 M borate/1% BSA, pH 9.3 (to neutralize the fractions). After evaporation of the acetonitrile, the fractions were lyophilized and then reconstituted in 1 ml of 0.2 M glycine/0.25% BSA, pH 8.8. Proinsulin, conversion intermediates and insulin in the fractions were quantified by radioimmunoassay. An equivalent volume of lyophilized HPLC eluate handled in parallel was used for the standard curve in order to compensate for any non-specific effect of residual salts in the fractions [39]. A standard of partially trypsin-treated human proinsulin containing proinsulin, des-31.32-split-proinsulin, des-64.65-split-proinsulin and fully processed insulin was subjected to HPLC after the samples to determine the elution times of the (pro)insulin-like products by absorbance at 213 nm. The standard was made by partial digestion of human proinsulin (1 mg/ml) in Tris/formate, pH 7.4, with trypsin (2 μg/ml; type XIII from Sigma) for 30 min at 30 °C and then with carboxypeptidase B (25 μg/ml; Boehringer-Mannheim) for 30 min at 30 °C [8,40]. The partially digested proinsulin was acidified by addition of 1 M HCl before injection on to an HPLC column.

Preparation of mutant proinsulin standard

As not all mutant proinsulins are co-eluted from HPLC columns with native proinsulin, it was necessary to prepare standards of these molecules. Transfected cells grown to near-confluence in two 10 cm-diameter Petri dishes were washed three times with Krebs/Ringer/bicarbonate buffer containing 10 mM Hepes, 0.5% BSA and 8.3 mM glucose, pH 7.4 (KRB/Hepes), preincubated for 15 min in KRB/Hepes at 37 °C, and labelled with 500 μCi of [^{3}H]leucine in 5 ml of KRB/Hepes per dish for 1 h. Cells were extracted in 5 ml of 1 M acetic acid/0.1% BSA, sonicated and centrifuged at 11000 *g* at 4 °C for 15 min. The supernatants were concentrated on C_{18} Sep-Pak cartridges and then immunoprecipitated with anti-(porcine insulin) serum using Protein A–Sepharose as the immobile support [41]. The immunoprecipitated products were displaced from the Protein A–Sepharose support with 1 M acetic acid/0.1% BSA, and repurified on C_{18} Sep-Pak cartridges to separate them from antibody (which was also displaced from Protein A by the acid). Unlabelled human proinsulin (200 μg) was added to the eluate as internal standard. One-tenth of the sample was subjected to HPLC at once in order to establish the elution time of radioactive products in the undigested material, and the remainder was partially digested with trypsin and carboxypeptidase B as described above and then injected on to the HPLC column.

Analysis of the kinetics of proinsulin conversion by pulse–chase experiments

Cells grown to near-confluence in 6 cm-diameter Petri dishes were washed three times with KRB/Hepes and preincubated for 15 min in KRB/Hepes at 37 °C. Cells were labelled with 200 μCi of [^{3}H]leucine in 2 ml of KRB/Hepes for 10 min and chased for up to 120 min in 2 ml of KRB/Hepes containing 1 mM unlabelled leucine. At the end of the chase period, cells were extracted into 2 ml of 1 M acetic acid/0.1% BSA, sonicated, centrifuged at 11000 *g* at 4 °C for 15 min and the supernatant was collected. Samples were concentrated on C_{18} Sep-Pak cartridges and then immunoprecipitated as described above. Radioactive (pro)insulin-like products were analysed by reverse-phase HPLC after addition of 10 μg of partially trypsin-treated human

proinsulin as internal standard (see above). Fractions of 1 ml were collected and the radioactivity was measured in a liquid-scintillation counter after addition of 3.5 ml of Lumaflow II scintillation cocktail.

RESULTS

Transfection of AtT20 cells with native or mutant human proinsulin cDNA

The mutant proinsulin cDNAs obtained by site-directed mutagenesis (Figure 1) were stably transfected into AtT20 cells and insulin-producing G418-resistant clones were screened by radioimmunoassay of products released from cells over 1 h under basal or stimulated conditions. Further experiments were performed on clones producing the most (pro)insulin-like immunoreactivity (i.e. more than 10 ng per 10^6 cells) and from which secretion could be stimulated 4–6-fold (Table 1). These are representative of fully regulated AtT20 cells releasing less than 5% of their (pro)insulin-like cellular content under basal conditions and exhibiting a significant response to secretagogues [28].

Cellular content of immunoreactive (pro)insulin-like products under steady-state conditions

To determine the distribution of immunoreactivity in the form of proinsulin, conversion intermediates and insulin in cells under steady-state conditions, cell extracts were subjected to HPLC and fractions radioimmunoassayed. Representative HPLC elution profiles for extracts of AtT20 cells transfected with native human proinsulin or the C1Pro mutant proinsulin are shown in Figure 2.

For cells transfected with native proinsulin cDNA the major immunoreactive peak was co-eluted with fully processed insulin (Figure 2a). The relative amounts of each (pro)insulin-like immunoreactive product were: 90% fully processed insulin and 10% proinsulin.

Cells transfected with C1Pro proinsulin exhibited a major peak that was co-eluted with des-64.65-split-proinsulin (the conversion intermediate in which cleavage has only occurred between C-peptide and the A-chain) and only a modest peak of fully

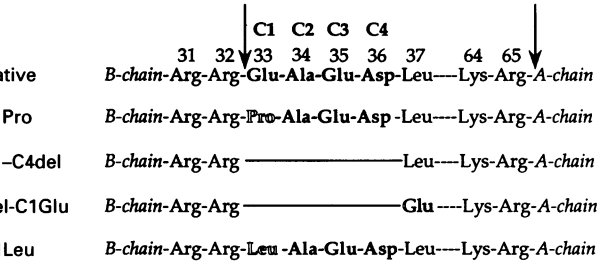

Figure 1 Amino acid sequence at the beginning of native and mutant human proinsulin C-peptides

The first four residues of the C-peptide (bold type) are numbered C1 to C4 and constitute the conserved acidic region. Numbering above each residue indicates its position in the full proinsulin molecule. Sites of cleavage during proinsulin conversion are shown by arrows.

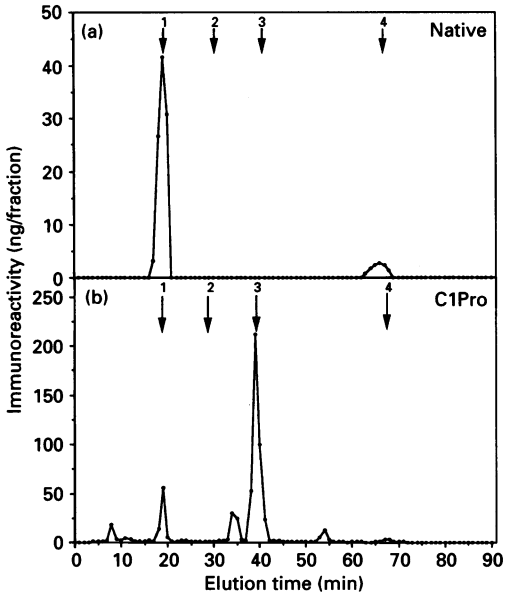

Figure 2 HPLC analysis of the steady-state cellular content of insulin-related peptides in AtT20 cells transfected with native (a) or mutant C1Pro (b) proinsulin

Confluent transfected AtT20 cells in 10 cm-diameter Petri dishes were extracted in acid. Cell extracts were analsyed by HPLC and the (pro)insulin-like immunoreactivity in the fractions was measured by radioimmunoassay. The elution times of human insulin (1), des-31.32-split-proinsulin (2), des-64.65-split-proinsulin (3) and proinsulin (4) are indicated by arrows.

Table 1 Cell content and release of immunoreactive insulin-related products from native, C1Pro, C1–C4del, del-C1Glu and C1Leu clones under basal or stimulated (IBMX/forskolin) conditions

Confluent monolayers of AtT20 cells stably transfected with native, C1Pro, C1–C4del, del-C1Glu and C1Leu proinsulin in six-well culture plates (35 mm-diameter wells) were incubated for 30 min in medium without (basal) or with (stimulated) 10 μM forskolin/1 mM IBMX. The media and cell extracts were analysed by radioimmunoassay. Data are presented as means ± S.E.M. for the number of independent experiments shown in parentheses on two individual clones per proinsulin species (except for del-C1Glu for which only one clone was studied).

	Insulin-like immunoreactivity				
Proinsulin...	Native (6)	C1Pro (9)	C1–C4del (9)	del-C1Glu (5)	C1Leu (10)
Basal secretion (ng/10^6 cells)	2.7 ± 1.0	1.2 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	1.6 ± 0.2
Stimulated secretion (ng/10^6 cells)	13.8 ± 5.7	7.6 ± 1.3	2.3 ± 0.2	3.5 ± 0.5	6.8 ± 0.7
Cell content (ng/10^6 cells)	121.0 ± 44.5	40.9 ± 7.1	27.2 ± 1.0	14.1 ± 1.9	77 ± 9.3
Basal secretion (% of cell content)	2.1 ± 0.1	3.3 ± 0.4	1.8 ± 0.2	4.5 ± 0.3	2.1 ± 0.1
Fold stimulation	4.1 ± 0.7	6.1 ± 0.7	4.9 ± 0.3	5.2 ± 0.2	4.4 ± 0.2

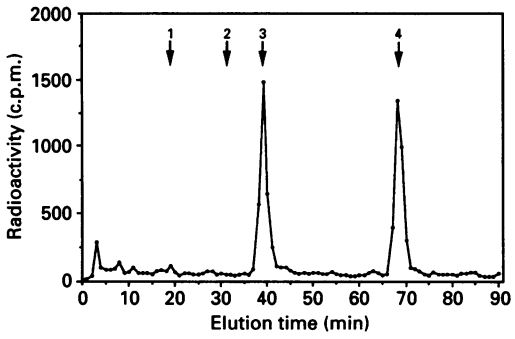

Figure 3 HPLC analysis of immunoprecipitable radioactive products in AtT20 cells expressing C1Pro mutant proinsulin after 10 min label and 60 min chase

Cell extracts were immunoprecipitated using anti-insulin serum, and precipitated products were subjected to HPLC. Radioactivity was measured in each fraction. The elution times of C1Pro insulin (1), des-31.32-split-proinsulin (2), des-64.65-split-proinsulin (3) and proinsulin (4) are indicated by arrows.

processed insulin (Figure 2b). The relative amounts of each (pro)insulin-like immunoreactive product were: 83% des-64.65-split-proinsulin, 15% fully processed insulin and 2% proinsulin. This indicates that the presence of proline at position C1 considerably slowed down cleavage at the B-chain/C-peptide junction.

The HPLC elution profiles for cells transfected with the other mutant proinsulins were quite similar to that shown for native proinsulin (Figure 2a). Thus cells transfected with C1–C4del, del-C1Glu or C1Leu proinsulin each showed a major peak of fully processed insulin accounting for more than 96% of the immunoreactivity, with only minor peaks of proinsulin or conversion intermediates. This indicates that the first four amino acids of the C-peptide were not absolutely required for cleavage at the B-chain/C-peptide junction and that a leucine at position C1 had a much less dramatic effect on cleavage than a proline.

From these data it is only possible to conclude that introducing a proline immediately C-terminal to the B-chain/C-peptide junction inhibits cleavage at this site. Measuring the relative amounts of immunoreactive proinsulin, conversion intermediates and insulin in the steady state does not thus provide any information on the kinetics of proinsulin conversion. For this it is necessary to follow the fate of newly synthesized proinsulin.

Kinetics of proinsulin conversion

Kinetics of proinsulin conversion were assessed by pulse–chase experiments (10 min labelling with [^{3}H]leucine and up to 120 min chase). Cell extracts, after prepurification and immunoprecipitation with anti-insulin serum, were fractionated by HPLC, and the radioactivity in the fractions was measured. A typical HPLC elution profile for C1Pro mutant proinsulin radioactive products is shown in Figure 3. Baseline and peak resolution shown for this particular mutant is representative of that found for the other mutants as well as for native proinsulin (reported by us previously [28]). Summing the radioactivity of the fractions in each peak (with correction for the relative number of leucines in insulin compared with proinsulin consequent on cleavage of C-peptide) allowed calculation of the percentage of each (pro)insulin-like product at each chase time and thus determination of the kinetics of proinsulin conversion.

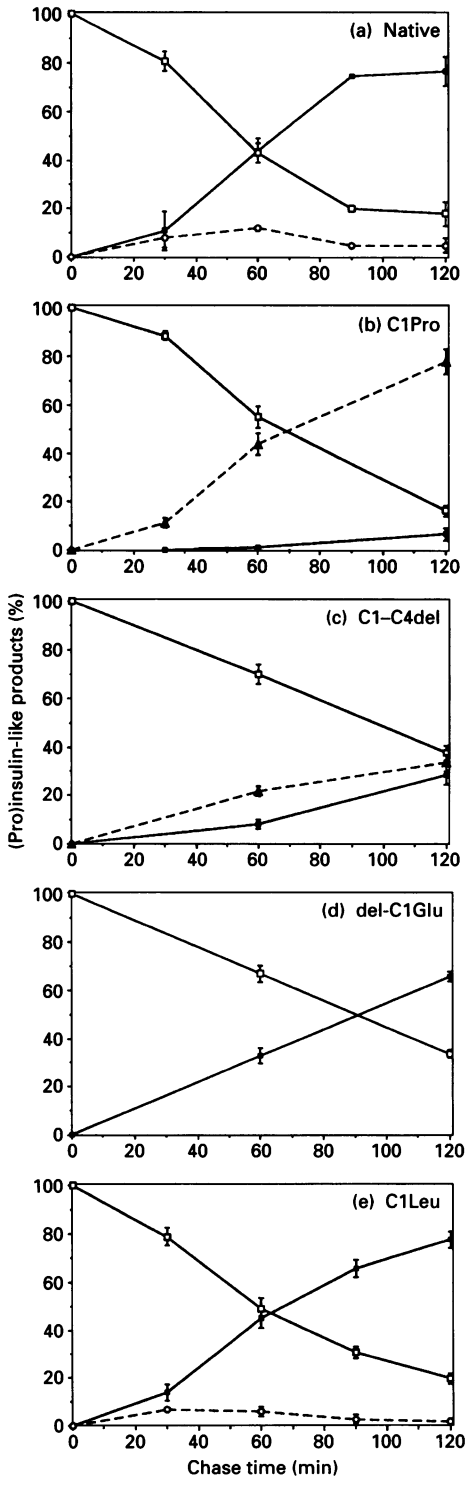

Figure 4 Time course of processing of native (a) and mutant (b, C1Pro; c, C1–C4del; d, del-C1Glu; e, C1Leu) proinsulin to conversion intermediates and insulin in transfected AtT20 cells

Transfected AtT20 cells grown to near-confluence in 6 cm-diameter Petri dishes were labelled for 10 min with 200 μCi of [^{3}H]leucine and chased for up to 120 min. Cell extracts were purified and fractionated by HPLC as described in the Materials and methods section and the radioactivity was measured in each fraction. The radioactivity eluted as insulin, des-31.32-split-proinsulin, des-64.65-split-proinsulin or proinsulin is expressed as a percentage of the sum of all four at each chase time. Each graph shows the mean ± S.E.M. for three independent experiments performed on two different clones for each mutant, except for del-C1Glu for which only one clone was used. □, Proinsulin; ○, des-31.32-split-proinsulin; ▲, des-64.65-split-proinsulin; ■, fully processed insulin.

Three pulse–chase experiments were performed for each proinsulin molecule under study.

For native human proinsulin, at the end of the 10 min pulse, all immunoprecipitable radioactivity was in the form of proinsulin. With time of chase, conversion occurred, with radioactivity associated with proinsulin decreasing in favour of conversion intermediates or fully processed insulin. Although proinsulin conversion is a complex multistep process, the time at which only 50% of immunoprecipitable radioactive products remain in the form of proinsulin provides a useful index of the rate of the initial cleavage event (whether it be at the B-chain/C-peptide or C-peptide/A-chain junction). By 55 min 50% of the native human proinsulin had disappeared, with progressive appearance of insulin and a transient accumulation of des-31.32-split-proinsulin (the conversion intermediate in which cleavage has occurred only between the B-chain and C-peptide) which accounted for approx. 15% of total insulin-related products after 60 min of the chase. No des-64.65-split-proinsulin could be detected. After 120 min of the chase, the major product of the conversion of native proinsulin was thus fully processed insulin, accounting for about 80% of the radioactivity with 15% residual proinsulin and 5% des-31.32-split-proinsulin (Figure 4a).

The time taken for disappearance of 50% of the C1Pro proinsulin was only slightly less than that for native proinsulin (68 min compared with 55 min) but the amount of the other (pro)insulin-like immunoreactive products was strikingly different (Figure 4b). There was a significant accumulation of des-64.65-split-proinsulin with only very small amounts of insulin even after 120 min of the chase. No des-31.32-split-proinsulin was detectable at any time.

The kinetics of conversion of C1–C4del proinsulin were intermediate between those of the native and C1Pro proinsulins. The rate of disappearance of proinsulin was much lower than that of native proinsulin (97 min for 50% radioactivity remaining as proinsulin). There was a progressive accumulation of des-64.65-split-proinsulin and fully processed insulin. After a 120 min chase period, there was 38% C1–C4del proinsulin, 33% des-64.65-split-proinsulin and 29% fully processed insulin (Figure 4c).

The time taken for only 50% of radioactivity to be left in the form of del-C1Glu proinsulin was also greater than for native proinsulin (91 min). Unlike in the conversion of the other proinsulins, neither conversion intermediate was detected at any time of the chase. After 120 min the major product of del-C1Glu proinsulin conversion was fully processed insulin, representing 63% of the radioactivity, with 37% proinsulin (Figure 4d).

Finally, the kinetics of conversion of C1Leu proinsulin were very similar to those of native proinsulin, with 50% radioactivity remaining as intact proinsulin at 60 min. By the end of the 120 min chase period, there was 78% fully processed insulin, 20% proinsulin and 2% des-31.32-split-proinsulin (Figure 4e).

DISCUSSION

In addition to any possible role as a peptide hormone after its secretion [42,43], C-peptide could play an additional or alternative role in either the targeting of proinsulin to secretory granules or its conversion into insulin. Although there is considerable sequence variation in the C-peptide from one species to the next [25,44], there are conserved sequences and domains, notably the acidic N-terminal region [25] which is the focus of the present study. Proinsulin that lacks this region is well targeted to granules [25], and, indeed, a mutant proinsulin in which the C-peptide was entirely missing was similarly targeted to granules in transfected AtT20 cells [45]. The finding that the C-peptide does not appear to play a significant role in targeting proinsulin to granules has directed interest to the study of its possible role in proinsulin conversion. In this context, it is suggested that C-peptide domains are involved in the correct presentation of the proinsulin junctions to conversion endoproteases. We have previously shown that proinsulin, in common with other proproteins, is cleaved more readily when the pair of basic residues at either junction is preceded by another basic residue in the -4 position [38,46–49]. In the rat, but not in man, one of these residues lies in the C-peptide (Arg^{62} of the rat proinsulins) demonstrating that a region or sequence in this peptide can indeed affect conversion.

It has been shown previously that deletion of the first four residues of the C-peptide, which constitute an acidic region largely conserved throughout evolution, inhibits conversion of rat proinsulin II in transfected AtT20 cells [25]. In this particular proinsulin molecule, the deletion introduced a proline as the new N-terminal residue of the C-peptide, immediately adjacent to the B-chain/C-peptide junction. As it has been shown that the presence of a proline C-terminal to a pair of basic residues blocks conversion of prorenin in transfected AtT20 cells [50], it remained unclear whether it was deletion of the acidic C-peptide region *per se* that had inhibited proinsulin conversion or the unacceptable presence of the proline C-terminal to Arg^{31}-Arg^{32} in the mutant proinsulin. The present study was intended to resolve this problem and to dissect in greater detail the precise role of the acidic residues in this region of the C-peptide in proinsulin conversion. As AtT20 cells express high levels of PC3 but only very low levels of PC2 [29–34], it is assumed that the results of this study reflect the substrate specificity of PC3 in proinsulin conversion. Given the differences in the levels of PC3 and PC2 in AtT20 cells and islet β-cells (the natural cellular setting for proinsulin conversion) [51,52], it is not necessarily justifiable to extrapolate the present findings to the latter cell type. It also remains possible that other members of the mammalian family of Kex-2-related proteases known to be present in AtT20 cells may also be active in proinsulin processing in these cells.

Conversion of prohormones is believed to occur predominantly in secretory granules [4–7]. A delay in conversion thus arises between the point of synthesis (the rough endoplasmic reticulum) and delivery of a prohormone to the conversion compartment (immature clathrin-coated granules). The rate of transit between the rough endoplasmic reticulum and granules differs from one protein to the next. For proinsulin it is relatively rapid [53], in keeping with a protein that is not glycosylated. A major change in three-dimensional structure may, however, affect this rate of transit. Furthermore, abnormal secretory proteins that are unable to assume their correct three-dimensional organization or that fail to oligomerize are in some way recognized in the rough endoplasmic reticulum and disposed of by a pre-Golgi degradation mechanism [54–57]. It has been shown for other proteins that mutations can lead to pre-Golgi degradation (see ref. [58] for review). In the present study we were not able to examine directly whether any of the mutant proinsulins are either transferred more slowly from the rough endoplasmic reticulum to granules than native proinsulin or possibly degraded in a pre-Golgi compartment (unfortunately our AtT20 cells expressing the highest levels of proinsulin still fall well short of the level required for such a quantitative evaluation of intracellular trafficking). Even if this were to occur, however, it is reasoned that there would not be any effect on the pattern of proinsulin conversion (i.e. the relative susceptibility of the two proinsulin junctions to cleavage), the topic of immediate interest in the present study.

Taken together, the data lead us to the following conclusions. The presence of a proline C-terminal to the B-chain/C-peptide

junction inhibits cleavage at this, but not at the C-peptide/A-chain, junction. Even though this residue, which is usually acidic (glutamate), can be replaced by a neutral amino acid (leucine) in the complete proinsulin molecule without affecting conversion, the N-terminal acidic tetrapeptide region of the C-peptide does appear to be important for facilitating cleavage at the B-chain/C-peptide junction. When this region is deleted, conversion is slowed down considerably. The replacement of leucine by glutamate immediately C-terminal to the B-chain/C-peptide junction of the deletion mutant can partially overcome this inhibition, suggesting that the presence of acidic residues in close proximity to the B-chain/C-peptide junction is important for its cleavage. Intriguingly, conversion even at the C-peptide/A-chain junction of the deletion mutant, and the deletion mutant with glutamate in the C1 position, but not the C1Pro mutant, seems to be slowed down. This implies that there is some interaction between the two junctions of the proinsulin molecule. This has been suggested by others on the basis of studies on the cleavage of the C-peptide/A-chain junction by PC2 showing that this enzyme favours des-31.32-split-proinsulin as its substrate over intact proinsulin [8,18,26]. Unlike these previous studies, however, our data which, as mentioned above focus on the action of PC3, suggest that in the intact proinsulin molecule cleavage by this enzyme at the C-peptide/A-chain junction is in some way facilitated by the presence of the acidic region of the C-peptide adjacent to the B-chain/C-peptide junction.

The complete three-dimensional structure of proinsulin has yet to be defined. One model of proinsulin based on an extrapolation from the structure of insulin itself does, however, suggest that the two junctions of proinsulin are probably in close spatial proximity [59]. All evidence favours the notion that the C-peptide is a relatively unstructured and flexible part of the proinsulin molecule [24,59]. One structural domain, the so-called 'C/A knuckle' has been identified by NMR analysis [24]. No function has yet been attributed to this domain, although it has been suggested that it may be involved in conversion [24]. On the basis of the present data and the remarkable conservation of this region of the C-peptide throughout evolution [25], it is now proposed that the N-terminal acidic region represents a second C-peptide structural domain which is clearly involved in proinsulin conversion.

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